The nature of the microfibrillar glycoproteins of elastic fibres

A biosynthetic study

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1. Cell cultures propagated from foetal bovine ligamentum nuchae synthesized and secreted two glycoproteins, designated MFP I and MFP II, that are closely related to elastic-fibre microfibrils. Glycoproteins MFP I (apparent mol.wt. 150 000) and MFP II (apparent mol.wt. 300 000) were metabolically labelled, separated from other culture-medium components by immunoprecipitation with a specific anti-(microfibrillar protein) serum, and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and sodium dodecyl sulphate/gel-filtration chromatography. 2. Ligament cells also synthesized and secreted fibronectin, but salt-fractionation and immunoprecipitation studies with a specific anti-(cold-insoluble globulin) serum established that neither glycoprotein MFP I nor glycoprotein MFP II was related to fibronectin. 3. The secretion of glycoprotein MFP I, but not that of glycoprotein MFP II, was enhanced by the addition of ascorbate to the culture medium. 4. Ascorbate-supplemented ligament cells incorporated [^3H]proline into glycoprotein MFP I, and 36% of the non-diffusible proline residues were hydroxylated, exclusively as 4-hydroxy[^3H]proline. Less than 1% of the total proline residues in [^3H]proline-labelled glycoprotein MFP II were hydroxylated. 5. Ascorbate-supplemented cells incorporated [^{14}C]lysine into glycoprotein MFP I and 30% of the non-diffusible lysine residues were hydroxylated. 6. Newly secreted glycoprotein MFP I was digested by highly purified bacterial collagenase to yield polypeptide fragments of apparent mol.wts. 50 000 and 30 000. Glycoprotein MFP II was not digested by bacterial collagenase. 7. The results suggest that elastic-fibre microfibrils are composed of a novel collagenous glycoprotein MFP I in association, as yet undefined, with a non-collagenous glycoprotein MFP II.

Elastic fibres are specialized connective-tissue structures that are largely responsible for the reversible extensibility of elastic ligaments and many compliant tissues such as lung and skin (Ross *et al.*, 1977; Fierer, 1977). Rope-like elastic fibres are a prominent histological feature of the well-developed neck ligament or ligamentum nuchae of ruminant animals, and this tissue has been particularly well studied in the bovine species (Greenlee *et al.*, 1966; Fahrenbach *et al.*, 1966; Cleary *et al.*, 1967; Wirtschafter *et al.*, 1967; Finlay & Steven, 1973; Kewley *et al.*, 1978).

The ultrastructural characteristics of elastic fibres are well established. They consist of an amorphous

core of the insoluble polymeric protein elastin and a peripheral mantle of microfibrils exhibiting a characteristic bead-like periodicity and a diameter of 10–12 nm (for a review see Ross, 1973). By studying the fibrous structures in bovine ligamentum nuchae during foetal development, Fahrenbach *et al.* (1966) and Greenlee *et al.* (1966) recognized that the accumulation of extracellular parallel bundles of microfibrils was an essential prerequisite for the deposition and polymerization of elastin, and hence the formation of mature elastic fibres.

The microfibrils are chemically distinct from the elastin in that they exhibit an affinity for cationic stains (e.g. uranyl acetate and lead citrate), whereas mature elastin displays a preferential affinity for anionic strains (e.g. phosphotungstic acid). Furthermore, chemical and enzymic extractions of bovine elastic fibres have separated the microfibrils from the polymeric elastin, and the amino acid analyses of the microfibrillar extracts were unlike those of either elastin or collagen (Ross & Bornstein, 1969, 1970).

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

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The enzymic extracts also contained hexosamines, thus intimating the presence of glycoproteins (Ross, 1973), a suggestion supported by the chemical extraction of glycopeptides from similar tissue (Serafini-Fracassini *et al.*, 1975). However, the microfibrillar material extracted from bovine ligamentum nuchae consists of a heterogeneous mixture of glycopeptides (Muir *et al.*, 1976; Sear *et al.*, 1977b), and has not proved suitable for the isolation and identification of native microfibrillar glycoproteins.

To facilitate the study of the biosynthesis of elastic-fibre components, we have developed techniques for the propagation of fibroblast cultures from foetal bovine ligamentum nuchae (Sear *et al.*, 1978). When cultured for several weeks, the ligament cells produce an extensive extracellular matrix in which 10–12 nm-diameter microfibrils and an amorphous elastin-like component coalesce to produce immature elastic fibres (Jones *et al.*, 1980). By analysing the spent medium from such cell cultures with the use of anti-(bovine microfibrillar protein) serum we identified, for the first time, two glycoproteins (designated MFP I and MFP II) that are related immunologically to the microfibrillar structures (Sear *et al.*, 1978). The present studies have been undertaken to define further the molecular natures of glycoproteins MFP I and MFP II.

It was of particular interest to examine the microfibrillar glycoproteins in relation to two other extracellular fibrous protein molecules, fibronectin and collagen. Early work on 'fibroblast surface antigen' suggested that a polypeptide (apparent mol.wt. 140 000) was closely related to a larger molecule (apparent mol.wt. 230 000) now known as fibronectin (for reviews see Vaheri & Mosher, 1978; Yamada & Olden, 1978). Since glycoprotein MFP I has apparent mol.wt. 150 000 (Sear *et al.*, 1978), it was important to determine whether this glycoprotein was related to fibronectin. Several different criteria were used to establish that glycoproteins MFP I and MFP II and fibronectin are three distinct molecular entities. Suggestions that microfibrils might be related to the collagens have come largely from electron-microscopic studies of extracellular fibre development during embryogenesis (Frederickson *et al.*, 1977). Our own ultrastructural studies revealed the frequent juxtaposition of microfibrils and collagen fibres in the extracellular space *in vitro* (Jones *et al.*, 1980), and prompted a search for 'collagenous' characteristics in glycoproteins MFP I and MFP II.

Experimental

Materials

Dulbecco's modification of Eagle's minimum essential medium, the completely chemically defined

medium MAB 87/3 (Gorham & Waymouth, 1965) and foetal bovine serum were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. L-[6-³H]Fucose (25 Ci/mmol), [5-³H]proline (29 Ci/mmol), [U-¹⁴C]proline (270 mCi/mmol) and [U-¹⁴C]lysine (342 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Highly purified bacterial collagenase (grade I) was obtained from Boehringer, Lewes, Sussex, U.K., and was shown to have negligible proteolytic activity towards a [³H]tryptophan-labelled substrate (Lee-Own & Anderson, 1975). The ultrafiltration membranes (type PM30) were purchased from Amicon, High Wycombe, Bucks., U.K. All other reagents and materials were obtained from sources previously described (Sear *et al.*, 1977a; Heathcote *et al.*, 1978).

The preparation and characterization of the rabbit anti-(bovine microfibrillar protein) serum has been described elsewhere (Kewley *et al.*, 1977; Sear *et al.*, 1978). The rabbit anti-(human cold-insoluble globulin) serum, and a similar antiserum preadsorbed with human serum, were generously provided by Dr. A. Vaheri, University of Helsinki, Helsinki, Finland. The IgG fraction of the goat anti-(rabbit IgG) serum was purchased from Miles Laboratories, Stoke Poges, Slough, Bucks., U.K.

Cell culture

Fibroblast cultures were propagated from freshly excised ligamentum nuchae of a foetal calf [approx. 135 days of gestation, as estimated by the forehead-rump length (Bogart, 1959)] as described elsewhere (Sear *et al.*, 1978; Jones *et al.*, 1980). Primary cultures were passaged at a split ratio of 1:8 as described previously (Sear *et al.*, 1977a), and the subcultures were fed with a growth medium consisting of Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% (v/v) foetal bovine serum. When the secondary cultures had attained the stationary phase of growth and contained 2.7×10^5 cells/cm² of growth area they were suspended (Sear *et al.*, 1977a) and stored frozen in liquid N₂. The cells used in the present experiments had undergone no more than nine population doublings from the original primary culture.

Incubation of cells

The growth medium was removed from ligament-cell cultures (75 cm²) that had reached the stationary phase of growth and replaced with the completely chemically defined medium MAB 87/3 (10 ml). This latter medium provided serum-free maintenance of stationary-phase ligament cells for extended periods, as previously found for human skin fibroblasts (Sear *et al.*, 1975, 1977a). After a preincubation period of 4–6 h at 37°C, the medium

was again replaced with fresh medium MAB 87/3 (10 ml), which was supplemented with L-[6-³H]-fucose (20 μ Ci/ml) or one of the other labelled compounds specified above under 'Materials'. When the cultures were labelled with [U-¹⁴C]lysine the incubations were performed in lysine-free Dulbecco's modification of Eagle's minimum essential medium. In experiments concerned with the biosynthesis of collagenous molecules the incubation medium also contained β -aminopropionitrile fumarate (50 μ g/ml) and/or L-ascorbic acid (50 μ g/ml).

At the end of the incubation period (24 h) the culture medium, containing labelled macromolecules, was decanted, centrifuged at 1200 *g* for 10 min at room temperature (22°C) to sediment any detached cells, and then supplemented with the proteinase inhibitors phenylmethanesulphonyl fluoride (1 mM) and *N*-ethylmaleimide (5 mM). Samples that were concentrated by precipitation with (NH₄)₂SO₄ were also supplemented with EDTA (tetrasodium salt) (25 mM).

Salt precipitation of macromolecules

Proteinaceous macromolecules secreted into the culture medium were concentrated by precipitation with (NH₄)₂SO₄ at 80% saturation at 4°C in the presence of proteinase inhibitors before further analysis. In some experiments high-molecular-weight proteins (predominantly fibronectin and collagen precursor polypeptides) were similarly precipitated with 30%-satd. (NH₄)₂SO₄.

Proteins remaining in solution after treatment with (NH₄)₂SO₄ at 30% saturation were prepared for immunoprecipitation studies by dialysis at 4°C against four changes of 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl, 0.2 mM-*N*-ethylmaleimide and 10 mM- ϵ -aminohexanoic acid. The dialysed samples were concentrated over an Amicon PM30 membrane to a volume equal to that of the culture medium from which it was derived.

Immunoprecipitation of macromolecules

Culture medium containing labelled macromolecules, or dialysed samples prepared therefrom, were added to the rabbit anti-(microfibrillar protein) serum in the proportions 10:1 (v/v) and incubated for 2 h at room temperature (22°C) followed by 16 h at 4°C. At this stage in some experiments the IgG fraction of an anti-(rabbit IgG) serum raised in goats was added in excess (as determined by preliminary titration experiments) in order to ensure the complete precipitation of rabbit IgG-antigen complexes. Immunoprecipitates were washed twice with 62 mM-Tris/HCl buffer, pH 7.5 (at 4°C), containing phenylmethanesulphonyl fluoride (1 mM) before further analysis.

SDS/polyacrylamide-gel electrophoresis

The nature of the labelled polypeptides synthesized by cultured ligament cells was examined by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). The preparation of the samples and their analysis on cylindrical gels were conducted as described previously (Sear *et al.*, 1977a, 1978), except that radioactive polypeptides were located in gels stained with Coomassie Brilliant Blue R by preparing slices (1.15 mm thick) from the frozen gel (Sear *et al.*, 1977a) and digesting each slice in 0.1 ml of a solution containing 99 vol. of H₂O₂ (30%, w/v) and 1 vol. of NH₃ (27%, w/w) for 12 h at 60°C. Scintillation fluid [5 vol. of toluene containing 2,5-diphenyloxazole (7.5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.15 g/l) and 2 vol. of methoxyethanol] (10 ml) was then added to each vial, and the radioactivity was measured after a further 12 h incubation in the dark at 4°C.

Direct comparison of the polypeptide compositions of several different samples was achieved by using SDS/polyacrylamide slab gels, which consisted of a separating gel (15 cm $\times$ 15 cm $\times$ 0.3 cm) containing 6.5% (w/v) acrylamide and a stacking gel (1 cm $\times$ 15 cm $\times$ 0.3 cm) containing 3% (w/v) acrylamide. Electrophoresis was performed in a water-cooled apparatus at 25 mA per gel slab. After fixation overnight in methanol/acetic acid/water (25:7:68, by vol.) the gel was dehydrated in dimethyl sulphoxide, impregnated with 2,5-diphenyloxazole for fluorography, dried and exposed to pre-flashed Kodak Royal X-Omat X-ray film XH-1 (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Gel filtration of macromolecules on SDS/agarose

The molecular size of newly synthesized microfibrillar glycoproteins was examined by gel filtration on SDS/agarose under reducing conditions. The chromatography was performed at room temperature (22°C) on a column (1.5 cm $\times$ 90 cm) of 6% agarose (Bio-Gel A-5 m). Descending elution was performed at approx. 5 ml/h with 50 mM-NH₄HCO₃, pH 8.0, containing 0.1% SDS, 1 mM-dithiothreitol, 20 mM- ϵ -aminohexanoic acid and 0.2% NaN₃. Fractions (approx. 1.4 ml or 2.3 ml) were collected and a sample of each was taken for measurement of radioactivity in either the scintillation fluid described in the preceding section or another comprising 2 vol. of toluene containing 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g/l) and 1 vol. of Triton X-100. When microfibrillar glycoproteins were labelled with [5-³H]proline, appropriate peak fractions were pooled, hydrolysed in 6 M-HCl for 24 h at 110°C and analysed for 4-hydroxy[³H]proline by the method of Juva & Prockop (1966). The recovery of radio-

activity from the SDS/agarose column was consistently greater than 95%.

Samples were prepared for chromatography in electrophoresis sample-preparation buffer (Laemmli, 1970). Typically, a protein precipitate prepared from culture medium (10 ml) was dissolved in sample-preparation buffer (0.8 ml), and a portion (0.7 ml) was subjected to chromatography. The remainder of the sample (0.1 ml) was examined by SDS/polyacrylamide-gel electrophoresis.

Blue Dextran 2000 was used to determine the void volume, and $^3\text{H}_2\text{O}$ (approx. $0.05\mu\text{Ci}$) the total column volume. For molecular-weight estimations the column was calibrated with mixtures of purified proteins, including chick tendon procollagen I pro- α -chains (mol.wt. 150 000), rat tail tendon collagen α - and β -chains (mol.wts. 98 000 and 196 000 respectively), bovine serum albumin (mol.wt. 68 000), ovalbumin (mol.wt. 45 000) and ribonuclease A (mol.wt. 13 700).

Results

Lack of identity between microfibrillar glycoproteins and fibronectin

When ligament-cell cultures were labelled with [^3H]fucose and the newly synthesized glycoproteins released into the culture medium incubated with the anti-(microfibrillar protein) serum, the immunoprecipitate contained two prominent [^3H]fucose-labelled species (Fig. 1, track B). These glycoproteins are closely related to elastic-fibre microfibrils (Sear *et al.*, 1978) and are designated glycoprotein MFP I (apparent mol.wt. 150 000) and glycoprotein MFP II (apparent mol.wt. 300 000). The immunoprecipitate contained traces of other glycoproteins of high molecular weight, but the species having a mobility similar to that of fibronectin was also present in the control precipitate prepared from culture medium and anti-(microfibrillar protein) serum that had been adsorbed with a bovine microfibrillar-protein extract (Fig. 1, track A). The anti-(microfibrillar protein) serum was thus shown to react specifically with glycoproteins MFP I and MFP II.

When the culture medium from [^3H]fucose-labelled ligament-cell cultures was incubated with the anti-(cold-insoluble globulin) serum, the immunoprecipitate contained a major labelled species of apparent mol.wt. 240 000, i.e. fibronectin (Fig. 1, track D). Only trace amounts of glycoproteins MFP I and MFP II were detected in this sample, and similar amounts of both glycoproteins were found in the control precipitate prepared from culture medium and anti-(cold-insoluble globulin) serum that had been adsorbed with serum (Fig. 1, track C). The anti-(cold-insoluble globulin) serum was thus shown to react specifically with fibronectin.

Electrophoretic analyses on slab gels (e.g. Fig.

Fig. 1. Fluorogram of extracellular [^3H]fucose-labelled glycoproteins synthesized by ligament cells and after immunoprecipitation with specific antisera

Approx. 100×10^6 ligament cells were labelled for 24 h with [^3H]fucose ($20\mu\text{Ci/ml}$). Equal portions of the culture medium were treated with specific antisera to produce immunoprecipitates that were analysed on a SDS/polyacrylamide slab gel as described in the text. The samples in tracks A–E are as follows: A, immunoprecipitate prepared with anti-(microfibrillar protein) serum previously adsorbed with a bovine microfibrillar-protein extract (Sear *et al.*, 1978); B, immunoprecipitate prepared with anti-(microfibrillar protein) serum; C, immunoprecipitate prepared with anti-(cold-insoluble globulin) serum previously adsorbed with serum; D, immunoprecipitate prepared with anti-(cold-insoluble globulin) serum; E, glycoproteins precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation. The mobilities of glycoproteins of interest are indicated as follows; 1, glycoprotein MFP II; 2, fibronectin; 3, glycoprotein MFP I.

1) also served to identify unambiguously fibronectin and glycoproteins MFP I and MFP II among the glycoproteins that may be precipitated from ligament-cell-culture medium by $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation (Fig. 1, track E). Previously, these identities had been tentatively made by comparing electrophoretic mobilities of glycoproteins separated on individual cylindrical gels (Sear *et al.*, 1978). Furthermore, the results illustrated by fluorography in Fig. 1 were confirmed by electrophoresing similar samples on cylindrical SDS/polyacrylamide gels and

Fig. 2. Fluorogram illustrating the differential solubility in $(\text{NH}_4)_2\text{SO}_4$ of the $[^3\text{H}]$ fucose-labelled glycoproteins newly synthesized and secreted by cultured ligament cells

Approx. 100×10^6 ligament cells were labelled for 24 h with $[^3\text{H}]$ fucose ($10 \mu\text{Ci}/\text{ml}$). Equal portions of the culture medium were treated with $(\text{NH}_4)_2\text{SO}_4$ as detailed below and the precipitates were analysed on a SDS/polyacrylamide slab gel as described in the text. The samples in tracks A–C are as follows: A, glycoproteins precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation; B, glycoproteins precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation; C, glycoproteins precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation after removal of precipitate B. The mobilities of glycoproteins of interest are indicated as follows: 1, glycoprotein MFP II; 2, fibronectin; 3, glycoprotein MFP I.

and released by cultured ligament cells, could be precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation (Fig. 2, track B). These conditions also precipitated glycoprotein MFP II, but not glycoprotein MFP I (Fig. 2, track B). However, after removal of the 30%-saturation- $(\text{NH}_4)_2\text{SO}_4$ precipitate, all the available glycoprotein MFP I was sedimented by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 80% saturation (Fig. 2, track C). Fibronectin and glycoprotein MFP I therefore exhibit significantly different solubilities in the presence of $(\text{NH}_4)_2\text{SO}_4$.

Influence of ascorbate on the biosynthesis of the microfibrillar glycoproteins

The possibility of a relationship between the microfibrillar glycoproteins and newly secreted collagenous molecules was firstly investigated in cultures that were supplemented with ascorbic acid, conditions that are known to stimulate the biosynthesis and secretion of collagen (Barnes, 1975; Levene & Bates, 1975). Ligament cells were incubated in the presence of $[^{14}\text{C}]$ proline under conditions of ascorbate deprivation or supplementation, and the newly synthesized and released $[^{14}\text{C}]$ -proline-labelled macromolecules precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation were subjected to electrophoretic analysis. Examination of the Coomassie Blue-stained gels showed that ascorbate produced a marked increase in the concentrations of the procollagens, procollagen-derived intermediates and tropocollagen molecules in the ligament-cell culture medium (Fig. 3a, gels D and F). When the gels were sliced and analysed for radioactivity it was found that there was 8–9 times as much $[^{14}\text{C}]$ -proline associated with the collagenous molecules released from the ascorbate-supplemented cultures as with the similar molecules derived from the ascorbate-deficient cultures (results not shown).

The newly secreted $[^{14}\text{C}]$ proline-labelled microfibrillar glycoproteins were separated from the bulk of the macromolecules in the culture medium by immunoprecipitation before electrophoretic analysis. The Coomassie Blue-stained gels showed that ascorbate produced a significant but small increase in the amount of glycoprotein MFP I secreted, and the ^{14}C -radioactivity profiles derived from the sliced gels showed that this increase was approx. 2-fold (Figs. 3b and 3c). The concentration of glycoprotein MFP II in the culture medium was not significantly altered by ascorbate supplementation (Figs. 3b and 3c).

Similar analyses of cultures that were supplemented with ascorbic acid and β -aminopropionitrile fumarate showed that the pattern of macromolecule biosynthesis and secretion was similar to that obtained by supplementation with ascorbate alone (Figs. 3a, 3b and 3d).

staining the separated polypeptides with Coomassie Blue (results not shown).

Electrophoretic analyses on slab gels showed that $[^3\text{H}]$ fucose-labelled fibronectin, newly synthesized

Fig. 3. SDS/polyacrylamide-gel electrophoresis of macromolecules secreted by ligament cells in the absence or presence of β -aminopropionitrile fumarate and/or ascorbate

Three replicate cultures containing approx. 25×10^6 ligament cells were labelled for 24 h with [14 C]proline ($1 \mu\text{Ci/ml}$) in the presence of foetal bovine serum (10%, v/v) either unsupplemented (C and D) or supplemented with ascorbic acid ($50 \mu\text{g/ml}$) (E and F) or with ascorbic acid ($50 \mu\text{g/ml}$) and β -aminopropionitrile fumarate ($50 \mu\text{g/ml}$) (G and H). Each sample of medium was divided into two equal portions. High-molecular-weight macromolecules were precipitated from one portion by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ (C, E and G), and an immunoprecipitate was prepared from the other by using the anti-(microfibrillar protein) serum (D, F and H). The polypeptide compositions of the precipitated samples were analysed on cylindrical SDS/polyacrylamide gels and compared with those of collagen I α - and β -chains from rat tail tendon (A) and procollagen I pro- α_1 - and pro- α_2 -chains from chick-embryo tendon (B). The gels were stained with Coomassie Blue (a) and then sliced and assayed for radioactivity as depicted above (b, c and d). Radioactivity profiles for gels C, E and G are not illustrated. The mobilities of glycoproteins of inter-

Demonstration of hydroxyproline and hydroxylysine residues in glycoprotein MFP I

Newly synthesized and secreted [^3H]proline-labelled macromolecules were precipitated by the anti-(microfibrillar protein) serum and chromatographed under reducing conditions on a SDS/agarose column. The resulting elution profile (Fig. 4a) exhibited a major symmetrical peak of apparent mol.wt. 150 000 and was identified as glycoprotein MFP I on SDS/polyacrylamide slab gels (Fig. 4b). Analyses of the glycoprotein MFP I peak fractions for hydroxy[^3H]proline showed that, of the total proline residues present in the glycoprotein, 34% were hydroxylated. The chromatographic profile also exhibited a relatively broad peak of apparent mol.wt. 250 000–300 000 (Fig. 4a), which consisted of a mixture of polypeptides, predominantly fibronectin and glycoprotein MFP II (Fig. 4b). When these fractions were analysed for hydroxy[^3H]proline less than 1% of the total proline residues were found to be hydroxylated (Fig. 4a).

The polypeptide analyses illustrated in Fig. 4(b) showed that the immunoprecipitates prepared from ligament-cell-culture medium and the anti-(microfibrillar protein) serum contained small amounts of several [^3H]proline-labelled species having mobilities intermediate between those of glycoproteins MFP I and MFP II. Electrophoretic analysis of standard proteins suggested that these contaminants were predominantly fibronectin and procollagen I pro- α_1 - and pro- α_2 -chains (results not shown). To decrease the amounts of these extraneous proteins and thus improve the specificity of the radiochemical assays for glycoprotein MFP I, immunoprecipitates were prepared from culture medium after the collagens and fibronectin had been precipitated out with $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation. Glycoprotein MFP II was not analysed further by these techniques, as a significant amount of this glycoprotein was lost during the $(\text{NH}_4)_2\text{SO}_4$ precipitation (Fig. 2).

When the [^3H]proline-labelled post- $(\text{NH}_4)_2\text{SO}_4$ immunoprecipitate was examined by SDS/agarose chromatography, the profile shown in Fig. 5(a) was obtained. The identity of the major peak was confirmed as glycoprotein MFP I by SDS/polyacrylamide-gel electrophoresis (Fig. 5b), and the hydroxy[^3H]proline content of the peak fractions was determined as 36% of the total imino acid complement by the method of Juva & Prockop (1966). In other experiments the [^3H]proline-labelled

est are labelled: 1, glycoprotein MFP II; 2, fibronectin; 3, glycoprotein MFP I; the position of the Bromophenol Blue tracking dye is indicated by the heavy arrow.

Fig. 4. Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5m) of ^3H -labelled proteins precipitated from ligament-cell-culture medium with a specific anti-(microfibrillar protein) serum

Approx. 25×10^6 ligament cells were labelled for 21 h with $[5\text{-}^3\text{H}]$ proline ($20 \mu\text{Ci/ml}$) in the presence of ascorbic acid ($50 \mu\text{g/ml}$) and β -aminopropionitrile fumarate ($50 \mu\text{g/ml}$). An immunoprecipitate was prepared from the culture medium by using the anti-(microfibrillar protein) serum and anti-(rabbit IgG) serum, and then analysed (a) by SDS/gel-filtration chromatography and (b) by SDS/polyacrylamide-slab-gel electrophoresis as described in the text. In (a) the elution positions of standards are indicated as follows: V_0 , Blue Dextran 2000; X, procollagen I pro- α -chains; Y, bovine serum albumin. ●, Total ^3H radioactivity; ○, 4-hydroxy ^3H proline. In (b) the mobilities of glycoproteins of interest are labelled: 1, glycoprotein MFP II; 2, fibronectin; 3, glycoprotein MFP I.

Fig. 5. Gel-filtration chromatography on SDS/agarose (Bio-Gel A-5m) of ^3H proline-labelled glycoprotein MFP I prepared by differential $(\text{NH}_4)_2\text{SO}_4$ fractionation and immunoprecipitation

Approx. 25×10^6 ligament cells were labelled for 21 h with $[5\text{-}^3\text{H}]$ proline ($20 \mu\text{Ci/ml}$) in the presence of ascorbic acid ($50 \mu\text{g/ml}$) and β -aminopropionitrile fumarate ($50 \mu\text{g/ml}$). The fraction precipitated by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ was removed from the culture medium, and an immunoprecipitate was prepared from the dialysed supernatant by using the anti-(microfibrillar protein) serum and the anti-(rabbit IgG) serum. The immunoprecipitate was analysed (a) by SDS/gel-filtration chromatography and (b) by SDS/polyacrylamide-slab-gel electrophoresis as described in the text. In (a) the elution positions of standards are indicated as follows: V_0 , Blue Dextran 2000; X, procollagen type I pro- α -chains; Y, bovine serum albumin. ●, Total ^3H radioactivity; ○, 4-hydroxy ^3H proline. In (b) the mobilities of glycoproteins of interest are labelled: 1, glycoprotein MFP II; 2, fibronectin; 3, glycoprotein MFP I.

Fig. 6. Determination by ion-exchange chromatography of the proportions of 4-hydroxyproline and proline in newly synthesized and secreted glycoprotein MFP I. An experiment similar to that illustrated in Fig. 5 was performed, and the glycoprotein MFP I peak fractions obtained by SDS/gel-filtration chromatography were hydrolysed in 6M-HCl at 110°C for 24 h in a sealed tube. The hydrolysate was evaporated to dryness, dissolved in 1 ml of 7 mM-sodium citrate buffer, pH 3.0, and applied to the long column of a JEOL JLC 6AH automatic amino acid analyser, which was eluted with the 7 mM-sodium citrate buffer, pH 3.0. A sample (0.95 ml) of each fraction (1.1 ml) was counted for radioactivity in the Triton-containing scintillation fluid described in the Experimental section. The elution positions of the following amino acids are indicated: 1, 3-hydroxyproline; 2, 4-hydroxyproline; 3, proline.

glycoprotein MFP I peak fractions were examined with an amino acid analyser, when 35% of the total imino acid residues were identified as 4-hydroxy- ^3H proline (Fig. 6). It is noteworthy that no 3-hydroxy ^3H proline was detected in the glycoprotein MFP I peak (Fig. 6).

Post-(NH_4) $_2\text{SO}_4$ immunoprecipitates were also prepared from cultures that were labelled with ^{14}C lysine. SDS/agarose chromatography of such samples showed that glycoprotein MFP I was the major ^{14}C lysine-labelled species present (Fig. 7a). When the glycoprotein MFP I peak fractions were subjected to acid hydrolysis and applied to the short column of the amino acid analyser, 30% of the total lysine residues were found to be hydroxyated (Fig. 7b).

Susceptibility of microfibrillar glycoproteins to digestion with bacterial collagenase

Ligament-cell cultures were incubated in the presence of ^3H fucose, and the newly synthesized and secreted microfibrillar glycoproteins were precipitated from the culture medium with the anti-(microfibrillar protein) serum. Duplicate samples of the immunoprecipitate were incubated in the pres-

ence or absence of bacterial collagenase and then analysed by slab gel electrophoresis (Fig. 8a). The digested sample was shown to contain approximately the same amount of glycoprotein MFP II as the undigested sample, but the band of radioactivity corresponding to glycoprotein MFP I was markedly decreased in intensity after the enzyme treatment. The digested sample also contained three ^3H -fucose-labelled glycopeptides that were not detected in the undigested sample, i.e. species of apparent mol.wts. 160 000–170 000, 50 000–60 000 and 30 000–40 000.

Similar digestion experiments were performed with immunoprecipitates prepared from culture medium that had been depleted of collagenous molecules by treatment with (NH_4) $_2\text{SO}_4$ at 30% saturation (see above). The fluorogram illustrated in Fig. 8(a) shows that ^3H proline-labelled glycoprotein MFP I was completely digested by bacterial collagenase, the digestion yielding small amounts of two ^3H proline-labelled polypeptides having apparent mol.wts. approx. 50 000 and 30 000.

Discussion

The inability of extraction techniques to yield homogeneous preparations of macromolecules derived from elastic-fibre microfibrils is due largely to the insoluble nature of elastic tissues. All the published attempts to purify the microfibrillar components have used enzymic or strongly dissociative conditions (Ross & Bornstein, 1969, 1970; Richmond, 1974; Serafini-Fracassini *et al.*, 1975), and have yielded glycopeptide mixtures of ill-defined structure and uncertain origin. Furthermore, the possibility that these glycopeptides may have been produced by non-specific proteolysis of structural macromolecules by tissue proteinases (Anderson, 1976) was not generally considered. Such problems can be largely overcome by culturing cells derived from elastic tissue and by studying the macromolecules secreted into the culture medium. This approach has provided much information about both collagen precursors (for review see Burke & Ross, 1979) and the elastin precursor molecule, tropoelastin (Abraham *et al.*, 1974, 1977; Rosenbloom & Cywinski, 1976; Bressan & Prockop, 1977), but elastic-fibre microfibrils have not been extensively studied in cell-culture systems. However, the demonstration that cell cultures derived from foetal bovine ligamentum nuchae accumulate extracellular tracts of microfibrils and immature elastic fibres prompted the search for the soluble precursor constituents of the insoluble microfibrils (Sear *et al.*, 1978; Jones *et al.*, 1980). Newly secreted macromolecules of microfibrillar origin may be readily recognized by using a microfibril-specific antiserum (Kewley *et al.*, 1977; Sear *et al.*, 1978),

Fig. 7. Preparation of [^{14}C]lysine-labelled glycoprotein MFP I by SDS/agarose (Bio-Gel A-5 m) chromatography, and the analysis of its [^{14}C]lysine residues by ion-exchange chromatography

(a) Approx. 50×10^6 ligament cells were labelled for 24 h with [$\text{U-}^{14}\text{C}$]lysine ($0.5 \mu\text{Ci/ml}$) in lysine-free culture medium (Heathcote *et al.*, 1980) supplemented with ascorbic acid ($50 \mu\text{g/ml}$) and β -aminopropionitrile fumarate ($50 \mu\text{g/ml}$). The fraction precipitated by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ was removed from the culture medium. The anti-(microfibrillar protein) serum and anti-(rabbit IgG) serum were then added to precipitate glycoprotein MFP I, which was further purified by SDS/agarose-gel-filtration chromatography under reducing conditions as described in the text. The elution position of standards are labelled: V_0 , Blue Dextran 2000; X, procollagen I pro- α -chains; Y, bovine serum albumin. (b) Fractions (1.4 ml) containing the peak of glycoprotein MFP I [nos. 59–61 in (a)] were pooled, hydrolysed in 6M-HCl at 110°C for 24 h and analysed on the short column of a JEOL JLC 6AH automatic amino acid analyser. Each fraction (0.8 ml) was counted for radioactivity in the Triton-containing scintillation fluid described in the Experimental section. The elution positions of standards are labelled: 1, hydroxylysine; 2, lysine.

and their proteolytic degradation can be markedly decreased by including proteinase inhibitors during all stages of subsequent chemical analyses.

Our biosynthetic experiments have established that ligament cells synthesize and secrete fibronectin *in vitro* (Fig. 1), a finding first suggested by the observation of fibronectin-like fibrils in the extracellular space of ligament-cell cultures (Jones *et al.*, 1980). The possibility of a chemical relationship between the microfibrillar glycoproteins and fibronectin, as alluded to in the introduction, was therefore strengthened. However, the comparative immunoprecipitation studies illustrated in Fig. 1 showed that the anti-(microfibrillar protein) serum is directed against antigenic determinants that are distinct from those exhibited by fibronectin, and similarly the anti-(cold-insoluble globulin) serum is directed against determinants that are distinct from those exhibited by glycoproteins MFP I and MFP II. Both glycoprotein MFP I and glycoprotein MFP II are therefore immunologically distinct from fibronectin.

When the solubilities of the microfibrillar glycoproteins and fibronectin were investigated, it was found that $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation precipi-

tates fibronectin from ligament-cell-culture medium (Fig. 2). Similar findings have been reported with spent medium from cultures of human skin fibroblasts (Sear *et al.*, 1977a), calf endothelial cells (Macarak *et al.*, 1978) and amniotic-fluid cells (Crouch *et al.*, 1978). Glycoprotein MFP I was readily distinguished from fibronectin, as it was soluble in $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation, but glycoprotein MFP II was precipitated (although to a variable extent) under these conditions (Fig. 2). The chemical differences between glycoprotein MFP I, glycoprotein MFP II and fibronectin are also clearly reflected in their different mobilities in SDS/polyacrylamide gels (Figs. 1 and 2). The immunological and chemical evidence therefore establish glycoproteins MFP I and MFP II as molecular entities distinct from fibronectin.

There have been several reports of a close spatial association between 10–20 nm-diameter microfibrils and collagen fibres (Frederickson *et al.*, 1977; Serafini-Fracassini *et al.*, 1977; Jones *et al.*, 1980). Such an association might be promoted if there were a structural relationship between the microfibrillar glycoproteins and collagen. The intriguing similarity in apparent molecular weight of glycoprotein MFP I

Fig. 8. Fluorograms illustrating the effect of bacterial collagenase on microfibrillar glycoproteins

In two separate incubations approx. 25×10^6 ligament cells were labelled for 24 h with either (a) $[6\text{-}^3\text{H}]\text{fucose}$ ($20 \mu\text{Ci/ml}$) or (b) $[5\text{-}^3\text{H}]\text{proline}$ ($20 \mu\text{Ci/ml}$). Incubation (a) was performed with unsupplemented culture medium from which an immunoprecipitate was subsequently prepared as described in the Experimental section. Incubation (b) was performed in the presence of ascorbic acid ($50 \mu\text{g/ml}$) and β -aminopropionitrile fumarate ($50 \mu\text{g/ml}$), and an immunoprecipitate was subsequently prepared from the culture medium depleted of the proteins precipitated by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ as described in the text. Both immunoprecipitates were suspended in 62 mM-Tris/HCl buffer, pH 7.0, containing 5 mM- CaCl_2 , 1 mM-phenylmethanesulphonyl fluoride and 5 mM-*N*-ethylmaleimide. Half of each sample was incubated with bacterial collagenase (enzyme/substrate ratio approx. 1:50, w/w) at 37°C for 4 h, and the other half was incubated without enzyme. Equal amounts of each sample were then analysed by SDS/polyacrylamide-slab-gel electrophoresis as described in the Experimental section. Tracks A and C contained the control (undigested) samples derived from incubations (a) and (b) respectively. Tracks B and D contained the samples derived from incubations (a) and (b) respectively that were incubated with bacterial collagenase. The mobilities of glycoproteins of interest are labelled: 1, glycoprotein MFP II; 2, glycoprotein MFP I.

and the interstitial collagen pro- α -chains (see Fig. 3, and Grant & Jackson, 1976) appeared to support this hypothesis. Supplementation of ligament-cell-culture medium with ascorbate produced an

approximately 2-fold increase in the amount of glycoprotein MFP I released into the medium (Fig. 3). Although this increase was smaller than the ascorbate-mediated stimulation of collagen synthesis (Fig. 3), it nevertheless raised the possibility that the enzymic hydroxylation of proline and/or lysine residues may play an important role in the biosynthesis and secretion of glycoprotein MFP I. The synthesis and secretion of glycoprotein MFP II were apparently unaffected by the presence of ascorbate in the culture medium (Fig. 3). These biosynthetic experiments complement electron-microscopic studies of ligament-cell cultures, in which ascorbate was observed to stimulate the production of extracellular collagen fibrils, but appeared to retard the formation of 10–12 nm-diameter microfibrils and to suppress elastogenesis (Jones *et al.*, 1980). In this context it is noteworthy that other workers have also reported that elastin biosynthesis appears to be depressed by ascorbic acid *in vitro* (Scott-Burden *et al.*, 1979; De Clerk & Jones, 1980).

The ascorbate-mediated stimulation of glycoprotein MFP I secretion suggested that this glycoprotein contained hydroxylated proline and/or lysine residues. Analyses of glycoprotein MFP I, labelled with $[^3\text{H}]\text{proline}$ under conditions of ascorbate supplementation, showed that approx. 36% of the radioactivity associated with the glycoprotein resided with 4-hydroxy $[^3\text{H}]\text{proline}$ (Fig. 6). There was a notable lack of 3-hydroxy $[^3\text{H}]\text{proline}$. Similar experiments with $[^{14}\text{C}]\text{lysine}$ -labelled glycoprotein MFP I showed that approx. 30% of the lysine residues were hydroxylated (Fig. 7). These results suggested that glycoprotein MFP I is chemically related to the collagens, and the susceptibility of the glycoprotein to bacterial collagenase (Fig. 8) supported this contention. However, the available evidence also suggested that glycoprotein MFP I was unlike any type of collagen or procollagen hitherto characterized. In particular, the solubility of glycoprotein MFP I in 30%-saturated $(\text{NH}_4)_2\text{SO}_4$ distinguishes it from type-I, -II and -III procollagen species, which are insoluble under such conditions (Olsen *et al.*, 1976; Harwood *et al.*, 1977; Scott *et al.*, 1977; Uitto, 1977). This observation suggests that glycoprotein MFP I is much more highly glycosylated than are the interstitial collagen precursors, a possibility that is supported by several other findings, including the ease with which glycoprotein MFP I is labelled with $[^3\text{H}]\text{fucose}$, its avidity for the periodic acid-Schiff reagent (Sear *et al.*, 1978) and its high content of hydroxylysine (Fig. 7).

It is unlikely that glycoprotein MFP I is related to the type-IV collagen precursor, in view of the latter's lower mobility in SDS/polyacrylamide gels and significantly higher degree of proline-residue and

lysine-residue hydroxylation (Grant *et al.*, 1972; Heathcote *et al.*, 1978, 1980), as well as its insolubility in $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation (Crouch & Bornstein, 1979; Alitalo *et al.*, 1980; Alitalo, 1980). Furthermore, the absence of 3-hydroxyproline from glycoprotein MFP I (Fig. 6) also argues against its being related to type-IV collagen (Heathcote *et al.*, 1978) or to the 3-hydroxyproline-rich type-I procollagen associated with newly anchored fibroblasts (Lembach *et al.*, 1977; Schwartz *et al.*, 1979). Type-V collagen also appears to be more highly hydroxylated than is glycoprotein MFP I (for review see Bornstein & Sage, 1980) and, although we have detected this collagen in foetal bovine nuchal ligaments, other studies in our laboratory indicate that antiserum to bovine type-V collagen does not recognize glycoprotein MFP I (K. R. Knight & M. E. Grant, unpublished work). It is also noteworthy that a glycosylated elastin-like protein, initially designated as proelastin, and having a similar size to glycoprotein MFP I, has been detected in cultures of rabbit smooth-muscle cells (Foster *et al.*, 1978). On the basis of hydroxyproline content, hydroxylysine content and collagenase susceptibility, glycoprotein MFP I does not appear to be related to this high-molecular-weight species, nor can it be related to tropoelastin (mol.wt. 70000), which is non-glycosylated (Grant *et al.*, 1971).

The digestion of glycoprotein MFP I by bacterial collagenase yielded two glycopeptide fragments having apparent mol.wts. approx. 50000–60000 and 30000–40000 (Fig. 8) and that had very low contents of hydroxyproline (C. H. J. Sear, unpublished work). Since the undigested glycoprotein has apparent mol.wt. 150000 (Sear *et al.*, 1978), the digested 'collagenous' portion or portions of the glycoprotein MFP I molecule have a total molecular weight of approx. 50000–70000. This finding again distinguishes glycoprotein MFP I from all the known collagen or procollagen species (for a review of the general structure of the procollagens see Fessler & Fessler, 1978; Heathcote & Grant, 1980). The appearance in ligament-cell-culture medium of two [^3H]fucose-labelled glycopeptides having electrophoretic mobilities similar to those of the collagenase-generated species mentioned above (Fig. 1) suggests that a significant proportion of newly secreted glycoprotein MFP I molecules undergoes limited enzymic proteolysis *in vitro*. Collagenase digestion of some immunoprecipitates containing [^3H]fucose-labelled glycoprotein MFP I also yielded a glycopeptide species of mol.wt. approx. 160000–170000 (Fig. 8), the origin of which is unknown. Glycoprotein MFP II appears to have no 'collagenous' character, as evidenced by its lack of hydroxyproline residues (Fig. 4) and resistance to digestion by bacterial collagenase (Fig. 8).

The precipitation from ligament-cell-culture medium of two glycoproteins by the anti-(microfibrillar protein) serum raised the possibilities that glycoproteins MFP I and MFP II were chemically related or physically associated (Sear *et al.*, 1978). The present studies suggest that glycoproteins MFP I and MFP II are not chemically related (e.g. glycoprotein MFP II is not a dimerized form of glycoprotein MFP I). The anti-(microfibrillar protein) serum is clearly directed towards the antigenic determinants of glycoprotein MFP I, as this glycoprotein is quantitatively precipitated by the antiserum after virtually all the glycoprotein MFP II has been removed by salt fractionation (Fig. 2). It is not yet known whether the antiserum is also directed towards the antigenic determinants of glycoprotein MFP II or whether the precipitation of this glycoprotein in the presence of glycoprotein MFP I is due to an associative interaction between the two. That such an association exists is perhaps suggested by the variable extent to which glycoprotein MFP II can be separated from glycoprotein MFP I by 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ (Fig. 2).

The specificity of the anti-(microfibrillar protein) serum (Kewley *et al.*, 1977) implies that elastic-fibre microfibrils are composed of novel collagenous glycoprotein subunits, glycoprotein MFP I. A second, larger, non-collagenous glycoprotein, glycoprotein MFP II, may also be a structural component of the microfibrils, or it may be involved in the alignment and aggregation of glycoprotein MFP I monomers or the stabilization of the microfibrillar bundles. Our understanding of the mechanisms involved in the polymerization of glycoprotein MFP I and its interaction with elastin in elastic fibres must await more detailed structural studies of the microfibrillar constituents.

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